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## REMARKS/ARGUMENTS

Claims 1 to 12, 21 to 25, 31 to 37, and 39 to 43 are pending in the application. No claims have been amended, canceled, or added herein. Applicants respectfully request reconsideration of the rejections of record in view of the following remarks.

**Alleged Obviousness**

**A.** Claims 1 to 7, 9 to 12, 21 to 25, 31 to 37, and 39 to 43 have been rejected under 35 U.S.C. § 103(a) as allegedly obvious over U.S. Patent No. 4,460,683 (hereinafter "the Gloger patent") in view of either U.S. Patent No. 4,179,337 (hereinafter "the Davis patent") or Zapilsky, *et al.*, *Topics in Applied Chemistry*, 347-370 (1992) (hereinafter "the Zapilsky reference"). Applicants respectfully traverse the rejection because the present specification demonstrates that conjugating uricase to polyethylene glycol of the molecular weight range recited in the claims leads to unexpected and superior results relative to the molecular weight ranges described in the cited references. M.P.E.P. § 2144.05.

Applicants respectfully submit that conjugating uricase to polyethylene glycol of a molecular weight of 12,000 to 30,000 would not have been obvious to those of ordinary skill in the art at the time the invention was made because the cited references fail to teach or suggest the unexpected advantages of doing so. Applicants have surprisingly discovered that conjugating uricase to polyethylene glycol of a weight average molecular weight of 20,000 dramatically increases the circulating half-life of the conjugate relative to that of uricase conjugated to polyethylene glycol of a weight average molecular weight of 5,000. For example, Table 1 of Example 6 provides data demonstrating that the circulating half-life of uricase-PEG 20,000 was ***12-fold*** longer than that of uricase-PEG 5,000.

None of the cited references, when considered alone or in combination, describes or suggests the unexpected and surprising advantages of conjugating uricase to polyethylene glycol of a molecular weight of 12,000 to 30,000. For example, the Gloger patent merely describes uricase from *Candida utilis* and *Aspergillus flavus* and fails to describe or suggest conjugating the enzyme to polyethylene glycol.

The Davis patent describes the conjugation of peptides and polypeptides to polymers such as polyethylene glycol and polypropylene glycol, and states that preferred polymers are 500 to 20,000 daltons, and especially preferred polymers are about 750 to 5,000 daltons. (See col. 2, lns. 53 to 58). The experimental examples found in the Davis patent, however, only describe conjugation of polypeptides to polyethylene glycol of a molecular weight of 750 or 2,000. The patent fails to teach or suggest the effects that conjugating polypeptides to polyethylene glycol of molecular weight higher than 2,000 would have, much less describe any advantages of doing so.

The Zapilsky reference states that polyethylene glycol useful for conjugation to peptides and proteins has a molecular weight of 2,000 to 20,000, and the reference surveys prior publications in which the conjugation of polyethylene glycol to various polypeptides was described. (See page 348 and Table 1). One of the prior publications describes experiments in which a polypeptide was conjugated to polyethylene glycol of a molecular weight of 8,000; over 38 of the prior publications describe experiments in which polypeptides were conjugated to polyethylene glycol of a molecular weight of 5,000. Four of the prior publications describe experiments in which polypeptides were conjugated to polyethylene glycol of a molecular weight of 4,500; one of the prior publications describes experiments in which a polypeptide was conjugated to polyethylene glycol of a molecular weight of 4,000;

three of the prior publications describe experiments in which polypeptides were conjugated to polyethylene glycol of a molecular weight of 2,000; three of the prior publications describe experiments in which polypeptides were conjugated to polyethylene glycol of a molecular weight of 1,900; two of the prior publications describe experiments in which polypeptides were conjugated to polyethylene glycol of a molecular weight of 750; and one of the prior publications describes experiments in which a polypeptide was conjugated to polyethylene glycol of a molecular weight of 750.

Thus, of all the prior publications surveyed in the Zapilsky reference, only one describes experiments in which a polypeptide was conjugated to polyethylene glycol of a molecular weight of 12,000 to 30,000. Specifically, one reference describes the conjugation of alkaline phosphatase to polyethylene glycol of a molecular weight of 1,900; 4,000; 5,000; 8,000; or 20,000. The commentary describing the results of the experiments states that "[m]odification [of alkaline phosphatase] with higher molecular weight mPEG gave more deactivation than did modification with the lower molecular weight mPEG." (See the Zapilsky reference page 360, attached as Appendix A in which the quoted portion of Table 1 is highlighted). The Zapilsky reference thus fails to teach or suggest any advantages of conjugating polypeptides to polyethylene glycol of a molecular weight of 12,000 to 30,000, and, in fact, describes a disadvantage associated with conjugation of alkaline phosphatase to polyethylene glycol falling within this weight range.

None of the cited references, therefore, when considered alone or in combination, describe a single advantage of conjugating a polypeptide to polyethylene glycol of a molecular weight of 12,000 to 30,000 as compared to polyethylene glycol of a molecular weight less than 12,000. Doing so would thus not have been obvious to those of ordinary

skill in the art at the time the invention was made. Applicants accordingly, respectfully request withdrawal of the rejection.

**B.** Claims 1 to 5, 8 to 12, 21 to 25, 31 to 37, and 39 to 43 have been rejected under 35 U.S.C. § 103(a) as allegedly obvious over Chua, C., *et al.*, *Annals of Internal Medicine* 109:114-117 (1988) (hereinafter "the Chua reference") in view of either the Davis patent or the Zapilsky reference. Applicants respectfully traverse the rejection because the present specification demonstrates that conjugating uricase to polyethylene glycol of the molecular weight range recited in the claims leads to unexpected and superior results relative to the molecular weight ranges described in the cited references. M.P.E.P. § 2144.05.

As discussed above, Applicants have surprisingly discovered that conjugating uricase to polyethylene glycol of a weight average molecular weight of 20,000 dramatically increases the circulating half-life of the conjugate relative to that of uricase conjugated to polyethylene glycol of a weight average molecular weight of 5,000. None of the cited references, when considered alone or in combination, describes or suggests the unexpected and surprising advantages of conjugating uricase to polyethylene glycol of a molecular weight of 12,000 to 30,000.

The Chua reference describes conjugation of uricase from *Arthrobacter protoformiae* to polyethylene glycol of a weight average molecular weight of 5,000. The reference fails to teach or suggest the effects that conjugating polypeptides to polyethylene glycol of molecular weight higher than 5,000 would have, much less describe any advantages of doing so. The teachings of the Davis and Zapilsky references are discussed above.

None of the cited references, therefore, when considered alone or in combination, describe a single advantage of conjugating a polypeptide to polyethylene glycol of a

molecular weight of 12,000 to 30,000 as compared to polyethylene glycol of a molecular weight less than 12,000. Doing so would thus not have been obvious to those of ordinary skill in the art at the time the invention was made. Applicants accordingly, respectfully request withdrawal of the rejection.

**Conclusion**

Applicants believe that the foregoing constitutes a complete and full response to the Office Action of record. Accordingly, an early and favorable Action is respectfully requested.

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Jane Inglese

Jane E. Inglese, Ph.D.
Registration No. 48,444

Woodcock Washburn LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

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# 21

# Use of Functionalized Poly(Ethylene Glycol)s for Modification of Polypeptides

SAMUEL ZALIPSKY and CHYI LEE

## 21.1. INTRODUCTION

The unique properties of poly(ethylene glycol) (or PEG) and its general compatibility with polypeptide materials facilitated development of a variety of different applications of this polymer.[1–11] A marked proportion of these applications involve the use of covalently linked polypeptide–PEG adducts (reviewed elsewhere[5–11]). For example, a number of PEG-enzymes were shown to be useful as catalysts, soluble and active in organic solvents.[7] Due to the affinity to the upper phase of PEG/Dextran and PEG/salt two-phase systems, PEG-modified proteins were proven useful both as diagnostic tools[8] and in preparative separations of biological cells.[9]

Unquestionably, the properties of polymer–polypeptide conjugates *in vivo* were a significant reason for the substantial amount of research reported in the area during the last two decades. It has been repeatedly demonstrated that covalent attachment of multiple strands of PEG to proteins produces conjugates with dramatically reduced immunogenicity and antigenicity.[10] Such preparations also show great resistance to proteolytic digestion, and remain present in the bloodstream a considerably longer time than the parent polypeptides. These beneficial properties of modified polypeptides allowed development of a number of PEG-modified therapeutic proteins.[10] Slight to moderate modification of potent allergenic proteins with PEG can often be

SAMUEL ZALIPSKY and CHYI LEE • Enzon, Inc., South Plainfield, New Jersey 07080. *Present address for S. Z.*: Department of Chemistry, Rutgers–The State University of New Jersey, Piscataway, New Jersey 08855.

*Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, edited by J. Milton Harris. Plenum Press, New York, 1992.

sufficient to convert them into tolerance inducers and/or substantially reduce their allergenicity.[11]

Although, in some instances, hydroxyl end groups of the polymer can be used directly for covalent attachment of molecules of biological relevance,[5,12–14] in most cases suitable functionalization of the polymer prior to the conjugation is essential.[12]

Here we will review the methods for PEG functionalization and its covalent attachment to polypeptides. Attention will be given to comparison of different methods for preparation of PEG conjugates, properties of the linkages between the polymer and peptide components and their influence on biological/enzymatic activities of the conjugates. This review will not deal with formation of PEG–peptide conjugates that are built by stepwise addition of amino acid residues.[5] However, some of the methods for interlinking peptides and PEG, though originally devised for chemical and/or enzymatic protocols for peptide synthesis, are of general applicability and will be included in our discussion.

First, we will briefly go over the relevant properties of PEG itself, so that the rationale for its extensive use as a carrier for biological molecules will become apparent.

## 21.2. PROPERTIES OF PEG

Poly(ethylene glycol) is a polyether–diol with the general structure HO—$(CH_2CH_2O)_n$—H. For the purpose of modification of peptides and proteins the useful molecular weight range is 2,000–20,000 daltons.

The polymer has a wide range of solubilities. It is soluble in most organic solvents as well as in aqueous solutions.[5] For example, it is soluble in both water and dichloromethane, to such an extent that very concentrated solutions (> 50%) can be prepared. Most polypeptides, as a result of their conjugation with PEG, in addition to retaining and in some cases enhancing their water solubility, also acquire solubility in some organic solvents.[7]

It was shown that ethylene oxide-based oligomers (MW < 400) can be oxidized *in vivo* into toxic diacid and hydroxy acid metabolites through a process initiated by alcohol dehydrogenase[15]; however, toxicity of PEGs of molecular weights above 1000 daltons is very low.[16] Extensive toxicity studies on PEG-4000 showed that this polymer can be safely administered intravenously in 10% solution to rats, guinea pigs, rabbits, and monkeys at a dose level of 16 g per kg body weight.[17] This corresponded to at least 1000-fold the amount of the polymer present in PEG-purified factor-VIII doses normally administered in humans. It was also reported that when administered intravenously to humans, PEGs of molecular weight 1000 and 6000 are readily excreted mainly via the kidney.[18] Biological activities of PEG conjugates are typically dominated by the non-PEG part of a molecule.[19]

The polymer by itself, even with a molecular weight as high as $5.9 \times 10^6$, is a very poor immunogen. PEG-recognizing antibodies (specific to 6–7 oxyethylene units) could be generated in rabbits by administration of PEG-modified allergenic proteins together with Freund's complete adjuvant.[20] In the absence of Freund's



complete adjuvant, PEG-modified proteins do not induce formation of immunoglobulins against the polymer, and also reduce the response against antigenic determinants of a protein molecule.[10,21]

The polyether backbone of PEG is not degradable by mammalian enzymes. However, several types of bacteria can readily degrade the polymer up to Mn = 20,000. The topic of polyether biodegradation was recently reviewed by Kawai.[22]

The presence of PEG in protein solutions, even at high concentrations, in free or in conjugated form does not have any adverse effect on protein molecules.[2] The observation that a single point attachment of PEG to a peptide does not restrict the access of enzymes to a modified peptide residue was made by a number of investigators,[23–25] and was used for preparation of enzymatically altered polymer–peptide adducts. Generally, the conformation of peptides or proteins does not change as a result of covalent conjugation with PEG.[6,26]

It was recognized in one of the first papers dealing with PEG–proteins[27] that the key property of PEG as a protein modifier is its ability to bind molecules of water. It was also suggested[1] that the ability of the polymer to influence a structure of several molecular layers of water might be one of the causes of its appreciable exclusion effect, which might explain such phenomena as: (i) the ability of PEG to act as a protein precipitating agent,[2] (ii) repulsion of proteins from PEG-modified surfaces,[4] and (iii) reduced immunogenicity and antigenicity of PEG–protein conjugates.[10,21,27]

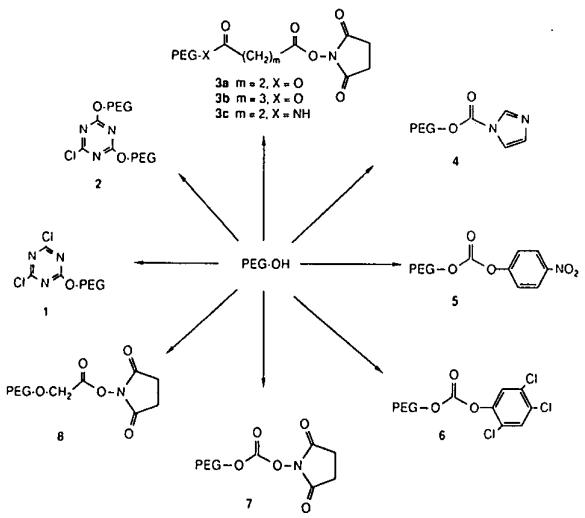
## 21.3. METHODS FOR COVALENT ATTACHMENT OF PEG TO POLYPEPTIDES

### 21.3.1. PEG Derivatives Reactive toward Amino Groups

Covalent attachment of PEG derivatives in the vast majority of cases has been achieved utilizing amino groups of polypeptide molecules as sites of modification. The first step in this process is substitution of the hydroxyl end-groups of the polymer by electrophile-containing functional groups. This process is often referred to as "activation." Derivatives of monofunctional polymers, capped on one end by methyl ethers (mPEG), were usually the reagents of choice for protein modifications.[10–12] Such modifications are expected to be free from crosslinking and result in the attachment of multiple strands of the polymer to the globular polypeptide core. A number of popular ways to activate the polymer are shown in Scheme 1.

#### 21.3.1.1. Most Commonly Used PEG-Based Reagents

In the original work of Davis and co-workers[27] trichloro-*s*-triazine (cyanuric chloride) was reacted with the primary alcohol groups on PEG so that only one of the chlorides is displaced from the triazine ring (**1**) and the remaining chlorides can be used for subsequent reaction with the amino groups of a protein. This approach was adopted by other investigators[8,9,28–30] and is still one of the most popular. The synthesis of **1** was recently optimized to assure the reproducible and complete




Scheme 1. Commonly used methods for activation of polyethylene glycol. For the purpose of this and the following schemes, the abbreviation PEG refers to the structures: RO—$(CH_2CH_2O)_n$—$CH_2CH_2$— or —$(CH_2CH_2O)_n$—$CH_2CH_2$—, where R is a simple alkyl residue, usually $CH_3$.

conversion of mPEG–OH to **1** as was proven by titration of chloride, $^{13}$C-NMR, GPC, and elemental analysis.[31] Although this approach provides a very effective one-step activation of the polymer, it suffers from various disadvantages such as toxicity of cyanuric chloride derivatives and excessive reactivity of **1** toward nucleophilic functional groups other than amines (e.g., cysteinyl and tyrosyl residues). In fact, cyanuric halides are considered to be the least suitable reagents for selective protein modification.[32] Consequently, modification of some proteins with **1** is often accompanied by a substantial loss of biological activity.[29,33–35]

A variation of the cyanuric chloride method in which two of the three chlorides originally present on the trichloro-*s*-triazine molecule are displaced by mPEG chains (**2**), and the remaining chloride used for the reaction with amino groups of a protein, was developed by Inada and co-workers.[7] Unfortunately, the communications describing the synthesis of **2** failed to provide proof of the structure of this activated polymer.[36–38] A recent report from the laboratory of Sehon[39] convincingly demonstrated lack of reactivity of the third Cl group on the triazine ring toward ethanolamine and ovalbumin, which places serious doubt as to whether 2,4-bis-(mPEG-*O*-)6-chloro- *s*-triazine (**2**) is capable of reacting with proteins. In light of this conflicting evidence, the issue of reactivity of **2** clearly needs to be clarified. It is interesting to mention that reactivity toward sulfhydryl groups reported for **1** by Wieder *et al.*[33] was also found for reagent **2**.[40]

The shortcomings of cyanuric chloride activation were overcome by using PEG-succinimidyl succinate (SS–PEG, **3a**). This form of activated PEG was first used for

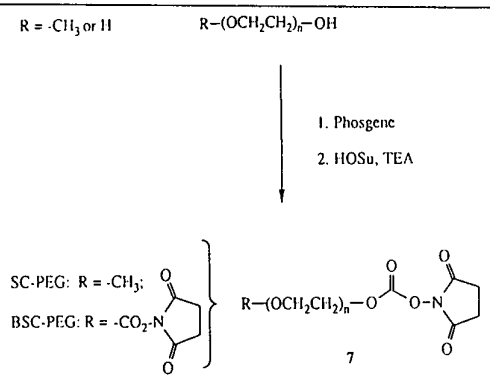


crosslinking of proteins[41] and synthesis of peptides substituted by PEG chains at their terminals[42] but was later adapted for preparation of mPEG–protein conjugates.[43] The reagent (**3a**) is usually prepared by succinylation of the terminal hydroxyl groups of PEG[14] followed by dicyclohexylcarbodiimide-mediated condensation with *N*-hydroxysuccinimide.[42,43] It reacts with proteins within a short period of time under mild conditions (30 min, pH < 7.8, 25 °C), producing extensively modified proteins with well-preserved biological activities.[26,43–45] The ester linkage between the polymer and the succinic ester residue has limited stability in aqueous media,[11,25,46] which causes slow hydrolytic cleavage of PEG chains from SS–PEG-modified proteins under physiological conditions. Substitution of the succinate residue in **3a** by glutarate (**3b**)[47] produced an activated form of the polymer very similar to SS–PEG, but with slightly improved resistance to hydrolysis of the ester linkage. Replacement of the aliphatic ester in **3a** by an amide bond (**3c**) improves the stability of the reagent and its conjugates even further.[46,48] However, preparation of **3c**, starting from hydroxyl terminated mPEG, involves 4 to 5 synthetic steps.[48]

Formation of urethane (carbamate) linkages between the amino groups of a protein and PEG overcomes the problem of hydrolytic release of the polymer chains. In fact, it was demonstrated on radioactively labeled PEG derivatives that urethane linkages are completely stable under a variety of physiological conditions.[49] The attachment of mPEG chains to polypeptides through carbamate linkages was first accomplished using imidazolyl formate derivatives of the polymer (**4**).[50] Several versions of a one-step synthesis of **4** using carbonyldiimidazole were reported.[35,51] Complete transformation of the hydroxyl groups of PEG was confirmed by both elemental analysis and NMR data. The polymer activated in this manner has a rather mild reactivity, and therefore long reaction times were required for protein modification (48–72 h, pH 8.5). However, good preservation of activity in these modified enzymes was usually observed.[35,50,52,53]

The products of protein modification using phenylcarbonates of PEG (**5** and **6**) also have PEG chains grafted onto the polypeptide backbone through urethane linkages.[54] Single-step activation protocols with commercially available chloroformates of 4-nitrophenol and 2,4,5-trichlorophenol were used for the preparation of **5** and **6**, respectively. These activated PEGs seem to react faster than **4**. However, both 4-nitrophenol and 2,4,5-trichlorophenol produced during the PEG- attachment process are toxic and hydrophobic molecules with affinities toward proteins.

Succinimidyl carbonates of PEG (SC–PEG, **7**), developed in our laboratory, constitute a further improvement in urethane-forming PEG reagents.[55,56] SC–PEG and its bifunctional analogs (BSC–PEG) of different molecular weights were prepared in a one-pot procedure as shown by Scheme 2. Polymeric chloroformate, generated *in situ* by treatment of PEG with phosgene, was reacted with *N*-hydroxysuccinimide (HOSu). Purified preparations of the SC-activated polymers were determined to contain the theoretical amounts of active carbonate groups. To estimate the reactivity of SC–PEG and to compare it to SS–PEG, measurements of hydrolysis and aminolysis rates of both activated polymers derived from mPEG-5000 were performed. The results of these experiments showed that SC–PEG is slightly less




**Scheme 2.** Preparation of succinimidyl carbonate derivatives of PEG. *Abbreviations:* HOSu, *N*-hydroxysuccinimide; TEA, triethylamine.

reactive yet a more selective reagent. Protein modification reactions with **7** can be performed within short periods of time (≈ 30 min) over a broad pH range, with the highest reactivity at pH ≈ 9.3. The HOSu released during polypeptide modification is a nontoxic material that is often used in bioconjugate and peptide chemistries as a leaving group residue. Our experience was that, unlike 4-nitrophenol and 2,4,5-trichlorophenol mentioned above, HOSu does not show affinity toward proteins and can be readily removed from the reaction solution by either dialysis or diafiltration. Under appropriate conditions BSC–PEG can be used as a homobifunctional cross-linker of proteins. It is also useful as a reactive macromonomer in polycondensation with diamines.[57]

The activated polymer **8** was prepared by carbodiimide-mediated esterification of carboxymethylated PEG with HOSu.[48,58,59] Carboxymethylation of PEG is one of the most straightforward ways to introduce carboxylic acid end groups onto the polymer. This is best accomplished by nucleophilic displacement of the bromide in ethyl bromoacetate with a PEG alkoxide, followed by saponification of the ester.[23,58–60] An alternative way to essentially the same derivative is by oxidation of the terminal hydroxyls of PEG,[48,61] but this process is often accompanied by polyether backbone degradation. A number of enzymes were modified using **8** with very good preservation of specific activity.[48,62,63] It was also used for modification of human hemoglobin.[46,59] The reactivity of **8** was reported to be one order of magnitude higher than that of **3**, as was estimated by hydrolysis ($t_{1/2}$ ≈ 1 min at pH 7.5, 27 °C) and aminolysis rates of the activated mPEGs.[64] The increased reactivity was explained by the presence of electron-withdrawing oxygen in one carbon-proximity to the carbonyl of the active ester in **8**. This result, combined with our own data (on SS– and SC–PEG)[55] and other literature sources,[54] allows us to estimate the order of reactivity of active acyl-bearing PEGs (assuming equality of all other variables, such as the molecular weight of the reagents) shown on Scheme 1:

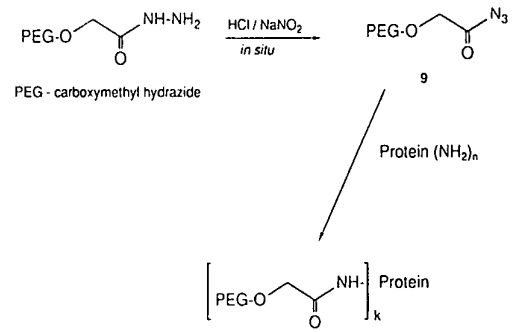
**8 >> 3a ≈ 3c > 3b ≥ 7 > 5 > 6 >4**



One has to bear in mind that the higher the reactivity of a reagent, the less likely it is to be selective, and consequently the higher is the probability of side reactions.

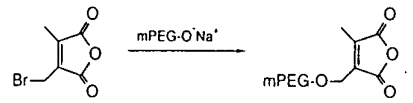
### 21.3.1.2. Less Commonly Used PEG Reagents for Modification of Amino Groups

In some instances another activated form of the polymer, PEG-carboxymethyl azide (**9**), was generated *in situ* from PEG–carboxymethyl hydrazide and then reacted with proteins according to the reaction shown in Scheme 3.[21,41,65] Normally, the slow reacting acyl azide functionality gets an additional boost of reactivity due to the stronger electrophilicity of the carboxymethyl residue.



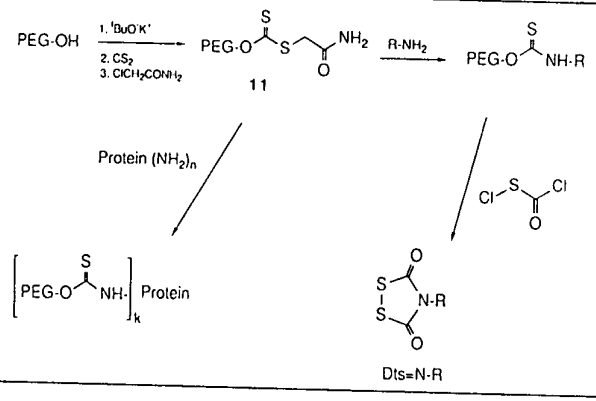

**Scheme 3.** Modification of polypeptides using *in situ* generated acyl azide.

Garman[66] reported synthesis of a polymeric analog of dimethylmaleic anhydride (**10**) suitable for PEG attachment to amino groups of polypeptides through a linkage which is slowly erodable under physiological conditions. The validity of this approach was demonstrated on plasminogen activator proteins. The PEG–plasminogen activator conjugates obtained by this method yielded active, completely deacylated proteins after 44 h incubation at pH 7.4, 37 °C.




**10**

Polyethylene glycols can be quantitatively converted into dithiocarbonate (xanthate) derivatives,[67,68] **11**, which were shown to be useful for grafting PEG chains onto proteins via thionourethane linkages (Scheme 4). The composition of PEGoxythiocarbonylated proteins could be conveniently determined spectrophotometrically from the increase in absorption at 242 nm as compared with that of native protein. The conjugates showed good chemical stability in a variety of aqueous buffers. Because of the mild reactivity of **11**, the modification reactions with bovine serum albumin and




**Scheme 4.** Preparation and use of PEGoxy-*S*-carboxamidomethyldithiocarbonate. R represents a peptide or an amino acid residue.

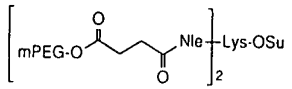
ragweed antigen E were carried out at pH range 9–10 for ≈ 20 h. (All the common free amino acids reacted quite readily with **11** within 30 min.) The xanthate **11** derived from bifunctional PEG-2000 proved useful as a polymeric reagent for the introduction of dithiasuccinoyl (Dts) protecting group into amino acids and dipeptides following the sequence of reactions shown on Scheme 4.[68]

Esters of organic sulfonic acids and PEG, *p*-toluenesulfonates (tosylates) in particular, have been useful as starting materials for the preparation of a variety of functionalized polymers.[68,69] Tresylates (2,2,2-trifluoroethanesulfonates) of PEG (**12**, PEG–$OSO_2CH_2CF_3$) were shown to be sufficiently reactive toward amino groups (≈ 100-fold more reactive than tosylates) to be considered useful as protein modifying reagents.[70] Modification of bovine serum albumin (BSA) with mPEG-tresylate in 16-fold excess to the amino groups, performed at pH 7.5, was complete in approximately 1 h and resulted in attachment of mPEG to 18 amino groups per protein molecule.[71] The comparison of these results to our own data on BSA modifications[55,56] using **3** or **7** leads us to believe that PEG–tresylate is the less reactive reagent. As a result of protein modification with PEG– tresylate, the polymer chains become grafted onto the polypeptide through very stable secondary amine bonds. There are two important consequences to this: (i) the total charge of the protein does not change in the process of modification; (ii) the modified proteins could be conveniently characterized by quantitation of lysine by amino acid analysis. The chemical composition of such conjugates can also be determined by the fluorescamine assay, which specifically measures primary amines and is not interfered with by the presence of secondary amino groups.[72] The assay based on the use of trinitrobenzene sulfonate (TNBS), which was extensively used for determination of the extent of modification in PEG–proteins,[73] theoretically should not be suitable in the case of PEG–tresylate-modified proteins. TNBS reacts with both primary and secondary amines as well as with other nucleophiles.[32] In light of this, it is surprising

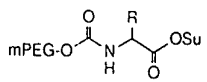


that the extent of modification of alkaline phosphatase modified with **12** was determined by TNBS assay.[35]

Although amino acid analysis has been used routinely for characterization of PEG–peptides,[5] it has only recently been recognized as a powerful analytical tool for characterization of PEG–proteins.[28,74,75] A single amino acid analysis run of a protein conjugate can provide several valuable pieces of information: (i) protein concentration, (ii) detection of possible side reactions that may occur during protein modification, and (iii) determination of the conjugate composition, provided that the linker is designed for this purpose. For example, the polymeric active ester **13**



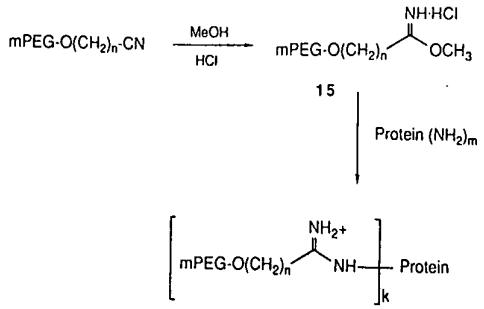

**13**



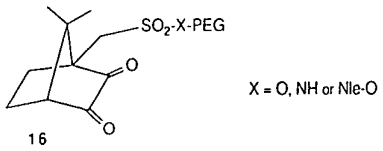

**14** R - side-chain residue of an amino acid

composed of two mPEG-5000 chains and containing reference amino acid norleucine (Nle) was specifically designed for convenient determination of the amount of PEG in its conjugates by amino acid analysis.[74] The amount of Nle determined in hydrolysates of proteins modified with **13** provides an accurate measure of the extent of modification. A similar rationale was used with regard to activated PEGs of general structure **14**.[75] Synthesis of PEGoxycarbonyl–amino acids, needed for preparation of **14**, can be readily accomplished by using active carbonates **5**–**7**[75] or by reacting isocyanato derivatives of amino acids with hydroxyls of PEG–OH.[68,76]

Imidoesters of mPEG (**15**) useful for protein modification were recently described in the patent literature.[77] These activated polymers were prepared by acid-catalyzed methanolysis of mPEG–cyanolkylethers and reacted with amino groups of proteins at pH 7–9 (Scheme 5). Making use of the fact that imidyl linkages survive the conditions of acidic protein hydrolysis, the extent of mPEG-amidination was determined by diminished lysine content in the conjugates. The advantage of this approach to protein modification is that the amidinated proteins possess the same net charge as the native ones.




**Scheme 5.** Preparation and use of mPEG–imidoesters.

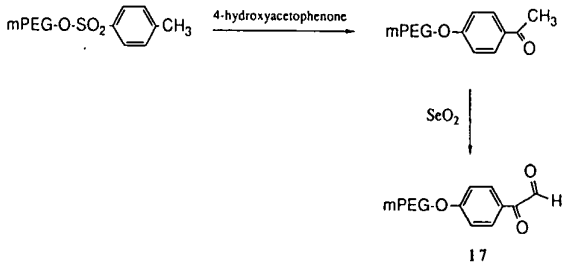



16

### 21.3.2. PEG Reagents Reactive toward Arginine Residues

Polymeric derivatives **16** for selective attachment of PEG to arginine residues were introduced to serve as carriers during semisynthesis of peptides in aqueous solutions.[78] The reagents were readily obtainable by reaction of camphorquinone-10-sulfonylchloride with PEG–OH, PEG–$NH_2$, or preferably with the norleucine ester of the polymer. The modified peptides were formed in borate buffer at pH 9.0, at 37 °C. They were stable to a variety of acidic and nucleophilic reagents, including hydroxylamine, the recommended agent for cleavage of cyclohexadione–arginine adducts. Active peptides could be released from the polymeric carrier by *o*-phenylenediamine. When norleucine was incorporated as a spacer between the camphorquinone moiety and the polymer, the extent of arginine modification was conveniently determined by amino acid analysis of hydrolysates of the PEG–peptides.

A variety of proteins were modified with a mPEG-5000 analog of phenylglyoxal (**17**),[79] a well-known reagent for modification of guanidino groups. The synthesis of **17** was performed according to the reactions depicted on Scheme 6. Readily obtainable mPEG–tosylate[68,69] was subjected to nucleophilic displacement by 4-hydroxyacetophenone, which was oxidized by selenium dioxide to yield mPEG–phenylglyoxal. Attachment of **17** to proteins through arginyl residues proceeded at pH range 5.5–9.3, at room temperature, and was measured by the decrease of arginine content in hydrolysates of modified polypeptides. The conjugates of **17** were claimed to possess very good stability and biological activity.




17

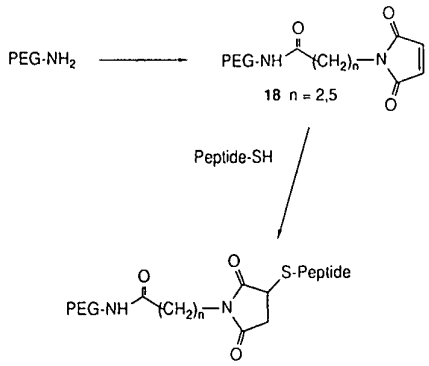
**Scheme 6.** Preparation of mPEG–phenylglyoxal.

### 21.3.3. PEG Reagents for Modification of Cysteine Residues

Maleimide-containing reagents are effective modifiers of free cysteinyl residues of polypeptides.[32] Preparation of PEG derivatives (**18**) bearing maleimido functional

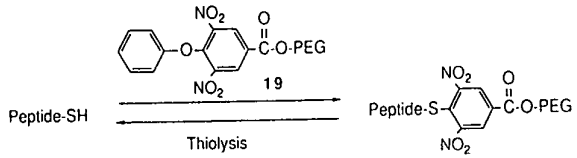


groups was reported using readily accessible[14,58] PEG–$NH_2$ and active esters of 6-maleimidohexanoic[80] and 3-maleimidopropionic[81] acids as starting materials (Scheme 7). Direct conversion of amino–PEG into a maleimido–PEG using maleic anhydride was also described.[21] A mutant protein of interleukin-2, in which cysteine at position −3 replaced the naturally occurring glycosylated threonine residue, was coupled with **18** ($n$ = 5) to produce a well defined, fully bioactive PEG–polypeptide.[80] The reagent **18** ($n$ = 2) was used for selective entrapment of free thiol-containing peptides on the polymer, in order to simplify purification of unsymmetrical cystine-peptides from a reaction mixture.[81]




**Scheme 7.** Preparation and use of PEG–maleimides.

Glass and co-workers reported preparation of 4-phenoxy-3,5-dinitrobenzoylPEG (**19**).[24] This PEG derivative reacted rapidly and selectively at neutral pH with




sulfhydryl groups of peptides to yield polymer–peptide adducts in which the components were linked by a thiol-sensitive dinitrophenylene linker. Thus it was possible to attach a peptide to the polymer and, after performing some chemical and/or enzymatic alterations on the conjugate, release the derivatized peptide from the PEG carrier.

### 21.3.4. Coupling of PEG Derivatives to Carboxylic Groups of Polypeptides

Amino–PEG was reacted with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-activated carboxyl groups of trypsin and other proteins.[21] The selectivity of such reaction is rather poor, due to the fact that amino–PEG has similar reactivity to the

lysiyl residues of proteins. In another variation of this reaction *p*-aminobenzylether of PEG was coupled to carboxyl groups of D-glucose 6-phosphate dehydrogenase by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.[82] The crude conjugate retained 22% of the original enzymatic activity. The aromatic amino groups on the polymer have a lower $pK_a$ than the amino groups of lysine residues and therefore would be expected to exhibit higher reactivity toward activated carboxyls under the acidic conditions of such reactions (pH 4.8–6.0). In both types of modification involving aliphatic and aromatic amino–PEGs, the composition of the conjugates was not determined.

In a recent report[83] bilirubin oxidase was modified by a two-step protocol. First, 1,4-diaminobutane was coupled to the carboxyl groups using water-soluble carbodiimide. Then, both the newly introduced amino groups and the original amines of the protein were modified with reagent **2**. The activity of PEG–bilirubin oxidase was approximately 30% that of the native enzyme. Unfortunately, the composition of the conjugate was not reported. When using carbodiimide-mediated coupling of an appropriate nucleophile to a protein, one has to be aware of possible side reactions, such as modification of tyrosyl and cysteinyl residues and the formation of *N*-acylurea derivatives.[32]

### 21.3.5. Coupling of PEG to Oligosaccharide Residue of Glycoproteins

We found only one reported case of this type of protein modification. In this example,[84] the specific reactivity of a carbohydrate residue of horseradish peroxidase was used for anchoring PEG chains. Mild oxidation of the carbohydrate portion of the enzyme molecule with sodium periodate resulted in the formation of six aldehyde groups. Bifunctional amino–PEG of molecular weight 20,000 was reacted with the oxidized peroxidase to form Schiff base links which were reduced by sodium borohydride. The conjugate produced retained 91% of the original specific activity and contained on average three PEG chains per glycoprotein molecule. In addition to water, it was soluble and active in tetrachloromethane, toluene, chloroform, and dimethylformamide.

## 21.4. RELATIONSHIP BETWEEN COUPLING CHEMISTRY AND BIOLOGICAL ACTIVITY OF PEG–POLYPEPTIDE CONJUGATES

Only a few attempts have been made to address the issue of the interrelationship between the chemistry of conjugation or activation of PEG and the biological activity of a particular PEG–polypeptide conjugate.[35,45,53,67,85] Therefore, in order to overview this topic we had to compare data published by a number of different research groups. We compiled the characteristics of selected PEG–protein conjugates in Table 1, showing the different functionalized polymers that were used to modify each



protein. While the data in Table 1 are presented as reported in the primary sources, the methods used by the various authors to determine the chemical composition of the modified proteins were often different. For example, several variations of TNBS assay, often used to determine the extent of modification of proteins, had been employed by the researchers. While most authors used the procedure of Habeeb,[73] which measures the total number of amino groups on a protein, in some cases[50,52,54,62,87] other versions of the TNBS assay, yielding a measure of the readily accessible amino groups only, were employed. The reader also has to be aware that in many cases conditions for modification reactions and design of biological/enzymatic assays also differed from one laboratory to another. Despite the above-mentioned drawbacks, several conclusions can be drawn from the data summarized in Table 1, which also illustrates some of the interesting properties and applications of PEG–polypeptide conjugates.

Proteins modified with cyanuric chloride activated PEGs (**1** and **2**) almost always possessed lower enzymatic activity than the same enzymes modified using alternative chemistries. This is most likely due to excessive reactivity and thus lack of selectivity of these reagents, which results in modification of nucleophilic groups other than amines. The pattern of asparaginase inactivation as a result of exposure to **1** or **2** provides the clearest illustration of this phenomenon and is consistent with the known reactivity of cyanuryl halides toward tyrosyl residues.[32] Inactivation of asparaginase by tyrosyl-modifying reagents is well documented.[102]

King *et al.* reported that PEG conjugates of Antigen E obtained via use of **1** had approximately one order of magnitude lower antigenic activity compared to those conjugates derived from **11**,[28,67] even though about the same numbers of amino groups were modified with both reagents. One might speculate that attachment of mPEG chains to nucleophilic residues other than lysyls, in the case of reagent **1**, could be the reason for the substantial difference in the antigenic activities. The lower antigenicity could actually be advantageous for potential therapeutic use in allergic patients, since greater amounts of the modified allergen may be used safely.

Yoshinaga and Harris[35] examined the activity of PEG–alkaline phosphatase conjugates obtained by four different coupling methods. The best preservation of activity was observed when SS–PEG (**3a**) was used as a modifying reagent, and only **1** caused substantial loss of enzymatic activity. The same research group examined alkaline phosphatase activity of conjugates obtained using cyanuric chloride-activated mPEGs and a number of its bifunctional analogs.[86] Interestingly, in contrast to the pattern observed with cyanuric chloride-activated mPEGs, protein modifications with the bifunctional PEGs resulted in better preservation of enzymatic activity which was independent of the extent of the modification or the molecular weight of the polymeric reagent used. It is pertinent to note that alkaline phosphatase in its active form is known to be a dimeric enzyme,[103] present in equilibrium with the only slightly active monomeric form. Therefore, the reported improved preservation of enzymatic activity could be attributed to partial fixation of the active dimeric form of the enzyme by the crosslinking of two monomers of alkaline phosphatase by the activated

*Table 1.* Chemical and Biological Characteristics of Selected PEG–Protein Conjugates

| Protein | Active PEG (MW)[a] | No. of PEGs per protein (% modified amino groups) | % Native enzyme activity (substrate) | Excerpts/applications | Ref. |
|---|---|---|---|---|---|
| Adenosine deaminase (ADA) | **1** | 9(40) | 28 | No reaction between PEG-ADA and the antibodies raised against native ADA. PEG–ADA may be suitable for treating human ADA deficiency because of long circulating life and the lack of detectable antibody formation in mice. | 34 |
| | **3a** | — | — | PEG–ADA was used successfully for the treatment of two children suffering from adenosine deaminase deficiency. Neither toxic effects nor hypersensitivity reactions were observed. | 44 |
| | **4** | 19(85[g]) | 76 | Long circulating half-life and significant retention of enzymatic activity in mice. However, no reduction of the immunogenecity was reported. | 52 |
| | **7** | 14(65[e]) | 51 | Used as a model for evaluation of the activated PEG. | 56 |
| Alkaline phosphatase | **1** | 19(88) | 33 | The reagent **1** caused significant loss of enzymatic activity. Best enzymatic activities were obtained in conjugates derived from **3a**. | 35 |
| | | 14(62) | 67 | | |
| | **3a** | 17(79) | 93 | | |
| | | 13(61) | 98 | | |
| | **12** | 17(77) | 86 | | |
| | | 16(73) | 82 | | |
| | **4** | 17(78) | 77 | | |
| | | 12(56) | 91 | | |
| | **1** | 5(23)–18(82) | 66–44 | Modification with higher molecular weight mPEG gave more deactivation than did modification with the lower molecular weight mPEG. Modification with bifunctional PEG gave highly active protein conjugates and there was little dependence on molecular weight or degree of modification. | 86 |
| | **1**(m1900) | 1(5)–19(86) | 97–55 | | |
| | **1**(4000) | 9(41)–17(77) | 72 | | |
| | **1**(8000) | 9(40)–16(72) | 70 | | |
| | **1**(20000) | 12(54)–18.5(84) | 80 | | |



| | | | | | |
|---|---|---|---|---|---|
| Antigen E | **1** | 8[f](44) | 1.0[h] | PEG–antigen E with reduced allergenic activities, yet retaining the immunogenic properties of antigen E. Substantially lower antigenicity was observed for conjugates obtained with reagent **1**. | 28 |
| | **1**(m2000) | 7[f](39) | 2.4[h] | | 67 |
| | **11** | 5.6[f](31) | 25[h] | | |
| | | 7.8[f](43) | 17[h] | | |
| | **11**(m2000) | 8.0[f](44) | 15[h] | | |
| Arginase | **6** | 55(60)[g] | 90 | The PEG–enzyme had an increased structural stability, a decreased digestion by proteolytic enzymes, and an expanded clearance time in rats. It is potentially useful for the therapy of arginine-dependent tumors or of familial hyperargininemia. | 87 |
| | **1** | 49(53) | 65 | Antisera from mice injected with native arginase reacted against arginase but not against PEG–arginase. In addition, antisera from animals injected with PEG–arginase reacted with neither native arginase nor PEG–arginase. | 88 |
| | **7** | 56(61) | 71 | Used as a model for evaluation of the activated PEG. | 56 |
| Asparaginase (*E. coli*) | **1** | 73(79) | 7(Asn) 15(ANA) | The modification of asparaginase with PEG(m5000) showed the reduction of the antigenicity and had a resistivity against trypsin. Interestingly, the asparaginase modified with PEG of 750 and 1900 daltons did not show a substantial change of the immunogenic properties. | 29 |
| | **1**(m1900) | 70(76) | 14(Asn) 25(ANA) | | |
| | **1**(m750) | 77(84) | 12(Asn) 20(ANA) | | |
| | **2** | 52(57) | 8(Asn) | The PEG–asparaginase conjugate has the same enzymatic properties ($K_m$ value and optimal pH) as the native enzyme. The half-lives of the modified and native enzymes in mice were 56 and 2.9 h, respectively. | 89 |
| | **2** | 30–48 (33)–(52) | 30–11 (Asn) | PEG–asparaginase conjugate retained 11% of the enzymatic activity but showed no binding activity toward anti-asparaginase serum. | 36 |
| | **3a** | 59(64) | 52(Asn) | PEG–asparaginase conjugates were active, stable, without immunosuppressive effect, and with extended half-lives in mice. | 43 |
| | **7** | 62(68[e]) | 54(Asn) | Used as a model for evaluation of the activated PEG. | 56 |
| | **8**[c](4500) | — | 8(Asn) | The modified asparaginase contained magnetite, which was used for facile removal of PEG–asparaginase from aqueous solution. | 90 |

(*continued*)

*Table 1. (Continued)*

| Protein | Active PEG (MW)[a] | No. of PEGs per protein (% modified amino groups) | % Native enzyme activity (substrate) | Excerpts/applications | Ref. |
|---|---|---|---|---|---|
| Chymotrypsin | 1 | 16(95) | 5(ATEE)<br>0(GFNA) | PEG–chymotrypsin retained catalytic activity and had increased solubility in organic solvents. It is useful for improving the coupling yield of peptide fragments and avoiding the risk of racemization. | 85 |
| | | 13(75) | 25(ATEE)<br>10(GFNA) | | |
| | 5 | 9(55) | 35(ATEE)<br>25(GFNA) | | |
| | 8 | 13(75) | 50(ATEE)<br>30(GFNA) | | |
| | 5(m2000) | 8.5–17<br>(50–100[g]) | 75–69<br>(BTNA) | The PEG–chymotrypsin was found to be soluble in benzene and DMF and catalyzed transesterification in cyclohexane. The enzymatic activity in organic solvents was decreasing with increasing the extent of modification and ranged 34–171% of the native activity. | 91 |
| | 6(m2000) | 2–7<br>(11–40[g]) | 77–64<br>(BTNA) | | |
| | 2 | 12.5(83) | 57(ATEE) | The yields of ester and amide formations both were 90% in 1,1,1-trichloroethane and the reaction rate was linearly enhanced with increasing amount of the PEG–chymotrypsin conjugate. | 92<br>38 |
| | 1 | 8(45) | 60(BTNA) | Substrate specificity of PEG–chymotrypsin in organic solvents was altered since arginine and lysine esters were found to be as effective as substrates as derivatives of aromatic amino acids. | 30 |
| | 7 | 9(54[e]) | 131(BTEE)<br>151(BTNA) | Used as a model for evaluation of the activated PEG. | 56 |
| | | 14(82[e]) | 122(BTEE)<br>161(BTNA) | | |
| Gulonolactone oxidase | 3a | 18(47[e]) | 74 | The modified enzyme was found to be more stable at 37 °C than native enzyme. The PEG–enzymes retained immunogenicity and reacted with preformed antibodies. The circulating half-life of the modified enzyme was not extended. | 45 |
| | 1 | 15(38[e]) | 67 | | |
| Lipase (*Pseudomonas fragi*) | 2 | (49) | 43[b] | The PEG–lipase catalyzed ester synthesis in organic solvents. The highest activity was observed in 1,1,1-trichloroethane. | 93 |
| | 8[c](4500) | — | 59–15[b] | The modified enzyme contained magnetite, which was used for convenient removal of PEG–lipase from reaction mixtures. The preparation was used for ester synthesis. | 90 |
| (*Pseudomonas fluorescens*) | 2 | (55) | 80[b] | PEG–lipase catalyzed ester synthesis, transesterification and aminolysis reactions in organic solvents. | 94 |
| | 2 | (52) | 67[d] | PEG–lipase was used in organic solvents with indoxyl acetate as a substrate; it was possible to determine the Michaelis-Menten constants for water. | 95 |
| | 2 | (60) | 70[b] | Two types of lipase were modified with two different reagents (**2** & **8**). In contrast to the case of *Pseudomonas fluorescens*, the enzyme from *Candida cylindracea* when modified with **8** catalyzed ester synthesis form short-chain alcohols and α- or β-substituted carboxylic acids in benzene. | 96 |
| (*Candida cylindracea*) | 8(m4500) | (47) | 56[b] | | |
| | 8(m4500) | (95) | 68[b] | The PEG–lipase catalyzed ester exchange reaction between dipalmitoyl phosphatidylcholine and eicosapentaeuoic acid. | 63 |
| Superoxide dismutase (SOD) (bovine erythrocyte) | 4 | 18 or 19<br>(90 or 95) | >95 | SOD coupled to PEG increased its plasma half-life from 3.5 minutes to 9 or more hours depending on the PEG derivative studied. | 50 |
| | 8 | 3(15)–18(90) | 90–72 | SOD–PEG conjugate showed longer half-life in rats than native SOD. | 48 |
| | 3c | 3(13)–18(90) | 90–70 | | |
| | 1 | 12(60)–14(70) | 100 | The PEG-modified enzyme increased cellular enzyme activities and provided prolonged protection from partially reduced oxygen species. | 97 |
| | 3a | — | 100 | | |
| | 1 | 19(95) | 51 | No evidence of an immune response to repetitive injections of PEG–SOD was observed. | 98 |
| | 6 | 10(50[g]) | 80 | The PEG-phenylcarbonate derivatives were stable in neutral aqueous solution and were reactive enough to modify proteins extensively in reasonable time periods. | 54 |
| | 5 | — | — | | |
| | 8 | 16(82[g]) | 75 | No structural modification occurred at the metal active site region and, in fact, the metal binding was higher in PEG–SOD than native SOD. The biological life of PEG–SOD decreased in the order i.v.> i.p.> i.m.> s.c. | 62 |
| | 3a | 11(57) | 47 | PEG-modified SOD resulted in high heterogeneity and substantial changes in isoelectric point and hydrophobicity. | 99 |

*(continued)*

*Table 1. (Continued)*

| Protein | Active PEG (MW)[a] | No. of PEGs per protein (% modified amino groups) | % Native enzyme activity (substrate) | Excerpts/applications | Ref. |
|---|---|---|---|---|---|
| Superoxide dismutase (SOD) (*cont.*) | | | | | |
| (Human) | **17** | 2.1 or 2.5 | | Arginine residues of SOD were selectively modified. | 79 |
| (Serratia) | **2** | 5(24) | 52 | The PEG (m5000) modified SOD showed enhanced anti-inflammatory activities | 100 |
| | **2**(m1900) | 10(48) | 41 | and radioprotective effects in mice. | |
| | **2**(m750) | 10(48) | 41 | | |
| | **2**(m350) | 10(48) | 39 | | |
| Tissue plasminogen activator | **4** | 10(44) | 80 | The reaction of rt-PA with activated-PEG **4** was much slower than the reaction | 53 |
| | | 13(60) | 30 | with activated-PEG **3a**. The conjugate of PEG-rt-PA has a potential to be used | |
| | | 16.5(75) | 20 | as thrombolytic agent in human. | |
| | **3a** | 12(55) | 36 | | |
| | | 14.5(66) | 14 | | |
| | | 22(100) | 0 | | |



| | | | | | |
|---|---|---|---|---|---|
| | **10** | 13(59) | 50–70 | Reversible conjugation of t-PA has been achieved with the PEG-contianing maleic anhydride reagent. | 66 |
| | **17** | 8(36) or 9(41) | | Arginine residues of t-PA were selectively modified. | 79 |
| Trypsin | **1** | 4(24) | 95(BAEE) | Proteolytic activity of the conjugate was markedly reduced. PEG–trypsin conju- | 101 |
| | | 9(59) | 150(BAEE) | gate (59% modified) dissolved soft blood clots at one-fourth the rate of trypsin. | |
| | **7** | 7(46[e]) | 95(BAEE) | Used as a model for evaluation of the activated PEG. | 56 |
| | | | 224(ZAPA) | | |
| | | 12(78[e]) | 92(BAEE) | | |
| | | | 326(ZAPA) | | |
| | **8** | 12.5(83) | 110(BAEE) | Used as a model for evaluation of the activated PEG. | 48 |

*Abbreviations*: ANA, Aspartic acid β-*p*-nitroanilide; Asn, Asparagine; ATEE, Acetyl tyrosine ethyl ester; BTEE, $N^{\alpha}$-benzoyl-L-tyrosine ethyl ester; BTNA, $N^{\alpha}$-benzoyl-L-tyrosine-*p*-nitroanilide; GFNA, Glutaryl phenyl alanine *p*-nitroanilide; ZAPA, $N^{\alpha}$-benzyloxycarbonyl-L-arginine-*p*-nitroanilide; rt-PA, Recombinant tissure plasminogen activator; i.v., intravenous; i.p., intraperitoneal; i.m., intramuscular; s.c., subcutaneous.

[a]Molecular weight given only for PEG derivatives different from mPEG-5000. Letter m appearing in parentheses prior to the number indicates mPEG derivative.

[b]Hydrolysis of olive oil in emulsified aqueous system.

[c]Bifunctional PEG-4500 was activated in presence of magnetite.

[d]Indoxyl acetate hydrolysis in emulsified aqueous solution.

[e]Fluorescamine assay (Ref. 72).

[f]Amino acid analysis.

[g]TNBS assay: version measuring only the readily accessible amino groups.

[h]% Antigenic activity of antigen E.

[i]The number of lysine residues modified were determined by a spectrophotometrical method and amino acid analysis.

bifunctional PEGs. Thus, the degree of this intermonomeric crosslinking, and not the number of modified amino groups, could have been the dominant factor determining enzymatic activity of a given preparation.

Superoxide dismutase (SOD) has been modified with PEG in a number of laboratories. With one exception,[99] the attachment of PEG through amide (reagents **3**, **8**) or urethane (reagents **4**, **6**) linkages caused only minimal inactivation of the enzyme. In the work of McGoff *et al.*,[99] which is the exception, the modified and the native enzymes were from two different sources. Consequently, the comparison of the two can hardly be valid. Interestingly, while Pyatak *et al.*[98] reported a 50% loss of SOD activity after modification with **1**, Beckman *et al.*[97] observed complete preservation of enzymatic activity of PEG–SOD derived from the same reagent. Unfortunately, no activity was reported for SOD derivatives obtained by attachment of **17** to arginyl residues of the protein.[79]

Preparation of functionally active, yet extensively modified, PEG conjugates derived from proteins having large-size substrates proved more difficult than with enzymes acting on low molecular weight substrates. For example, several PEG-tissue plasminogen activator (tPA) conjugates were prepared using succinimidyl succinate (**3a**) and imidazolyl formate (**4**) mPEGs as modifying reagents.[53] Preference was given to mPEG–imidazolyl formate-derived conjugates, due to their somewhat higher fibrinolytic activities. Regardless of the activated PEG employed, the activity of the conjugates decreased with increased extent of modification. Similarly to the case of tPA, proteolytic activity of PEG-modified trypsin was also drastically reduced[101] in contrast to the well preserved and in some cases even enhanced activity towards low molecular weight substrates (Table 1). Other proteolytic enzymes have shown similar behavior. Using far-ultraviolet circular dichroism and intrinsic protein fluorescence, Pasta *et al.*[26] showed that the serine protease, subtilisin, modified with **3a** maintains its native secondary structure and thus the integrity of its catalytic site. It is generally believed that steric hindrance is responsible for the diminished proteolytic activity of PEG-modified proteases. We believe that the well-documented ability of PEG to exclude proteins from its environment[1–4] is also partially responsible for this phenomenon.

From the examples shown in Table 1 the choice of the "best" performing activated PEG is not obvious. Overall, the acylating reagents (**3–8**) performed comparably well. In some cases the ease of preparation and shelf-life of reagents are very important considerations.[55] Based on these two criteria the urethane-forming functionalized PEGs (**4–7**) are clearly superior.

## 21.5. FUTURE PERSPECTIVES

Activity in the area of PEG-modified polypeptides has increased over the last two decades, as evidenced by growing numbers of research groups that have joined this field, as well as by the total number of relevant publications and patents. We expect this trend to continue during the nineties. It is clear that recombinant proteins, which have become more available and in many cases have potential for therapeutic use, can benefit from the increased stability, resistance to proteolysis, and extended plasma lifetime that conjugation with PEG is almost certain to provide. More sophisticated PEG-based reagents, that modify selective sites or residues of polypeptide molecules, will certainly emerge, accompanied by the development of the new analytical methods for the characterization of PEG–polypeptide conjugates. Superior quality of commercially available PEGs and their functionalized derivatives are already being developed by a number of companies. For example, the undesirable presence of bifunctional PEG contaminants in mPEG preparations[104] will have to be dramatically reduced to minimize the possibilities for crosslinking and heterogeneity of PEG–protein preparations. Some recently developed methods for selective introduction of one reactive functional group per polymer chain[60] as well as for synthesis of heterobifunctional PEG derivatives using readily available PEG–diols as starting materials[76] might minimize such complications and facilitate a more controlled and rationale design of PEG–polypeptide conjugates and their applications.[105]



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# 22

# Synthesis of New Poly(Ethylene Glycol) Derivatives

J. MILTON HARRIS, M. R. SEDAGHAT-HERATI, P. J. SATHER, DONALD E. BROOKS, and T. M. FYLES

## 22.1. INTRODUCTION

The chapters of this book describe the synthesis and use of a variety of active PEG derivatives designed to couple PEG to other materials. Despite the availability of these derivatives, there remains a need for new derivatives with presently unavailable properties and work continues in this area. Desirable properties include selectivity, stability, and ease of preparation. For example, it would be desirable to have derivatives that react with nucleophilic groups on proteins, but which do not react with water. Derivatives of a wide range of reactivities are always in demand. Similarly, there would be advantages to having derivatives that react with groups other than the commonly used amino groups. And, of course, the need for derivatives that can be prepared cheaply and easily in large quantity is critical for commercialization of the many biomedical and biotechnical applications of PEG chemistry.

Synthesis of new PEG derivatives is an on-going effort in our laboratories,[1-5] and here we describe three recent studies. The first applies solid-phase synthesis to prepare monodisperse PEGs, the second involves a new synthesis of PEG thiols and shows how this derivative can be used, and the third describes lab-scale synthesis of

J. MILTON HARRIS • Department of Chemistry, University of Alabama in Huntsville, Huntsville, Alabama 35899. M. R. SEDAGHAT-HERATI • Department of Chemistry, Southwest Missouri State University, Springfield, Missouri 65804-0089. P. J. SATHER and DONALD E. BROOKS • Departments of Pathology and Chemistry, University of British Columbia, Vancouver, British Columbia V6T 2B5, Canada. T. M. FYLES • Department of Chemistry, University of Victoria, Victoria, British Columbia V8W 2A2, Canada.

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